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Photon counting phosphorescence lifetime imaging with TimepixCam

Liisa M. Hirvonen,[1,a)] Merlin Fisher-Levine,[2,3] Klaus Suhling,[1] and Andrei Nomerotski[2]
[1]*Department of Physics, King's College London, Strand, London WC2R 2LS, United Kingdom*
[2]*Brookhaven National Laboratory, Upton, New York 11973, USA*
[3]*Department of Astrophysical Sciences, Peyton Hall, Princeton, New Jersey 08544, USA*

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TimepixCam is a novel fast optical imager based on an optimized silicon pixel sensor with a thin entrance window and read out by a Timepix Application Specific Integrated Circuit. The 256 × 256 pixel sensor has a time resolution of 15 ns at a sustained frame rate of 10 Hz. We used this sensor in combination with an image intensifier for wide-field time-correlated single photon counting imaging. We have characterised the photon detection capabilities of this detector system and employed it on a wide-field epifluorescence microscope to map phosphorescence decays of various iridium complexes with lifetimes of about 1 μs in 200 μm diameter polystyrene beads. © *2017 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).* [http://dx.doi.org/10.1063/1.4973717]

I. INTRODUCTION

The detection of single photons in the visible portion of the spectrum and the timing of their arrival with nanosecond resolution are important in many fields of science and technology, for example, in fluorescence spectroscopy and microscopy, Geiger mode lidar, optical tomography, and quantum cryptography.[1–4] In the life sciences, lifetime imaging in the microsecond regime is often used to image microenvironments, for example, oxygen concentrations or viscosities.[5,6] The long lifetime of the probe increases the sensitivity by giving the probe more time to interact with the environment and allowing short-lived autofluorescence from the sample to be discarded. Phosphorescence anisotropy measurements can also be used to study the rotational diffusion of large proteins, whose movement is too slow to be measured with fast nanosecond fluorescence decays.[7,8] Besides life sciences, phosphorescence lifetime imaging (PLIM) has been used to study air-flow and pressure in aerodynamic studies[9] and for temperature measurements in industrial thermometry applications.[10]

Time-correlated single photon counting (TCSPC) as a method to measure and map decay times has been reported to have the best signal-to-noise ratio of the standard time-resolved imaging methods.[11–14] It is independent of probe concentration or excitation intensity variations, and further advantages include a high dynamic range, high sensitivity, linearity, well-defined Poisson statistics, and easy visualization of photon arrival time data.[15,16]

Wide-field TCSPC requires the position of the arriving photon to be measured and recorded simultaneously with the arrival time. Microchannel plate (MCP) image intensifiers have been used for wide-field single photon detection since the 1960s, primarily for astronomical applications.[17] Early applications of single photon counting imaging in astronomy did not require any timing resolution; the amplified photon events were simply recorded with a slow camera, and the frames were added to form the final image.[18] With recent developments in complementary metal-oxide-semiconductor (CMOS) camera technology, these cameras can now reach MHz frame rates, allowing microsecond lifetimes to be measured directly with intensified CMOS cameras.[19,20] However, the achievable time resolution still depends on the frame rate and has yet to reach the nanosecond scale required for fluorescence lifetime imaging. Moreover, saving each full frame requires the management of large quantities of data.

Detection of individual photons and determination of their time of arrival is a promising route to measure the timing with nanosecond scale precision. This mode is already a widely used modality in X-ray imaging with semiconductor sensors,[21] where the signal is sufficiently large to detect individual photons directly and to enable measurement of their time and energy. High rate capabilities of the associated readout electronics allow fast accumulation of statistics so this information can be efficiently used for imaging. This approach requires the development of specialized CMOS ASICs (Application Specific Integrated Circuits), which are connected to a sensor through bump-bonding process, forming so-called hybrid pixel detectors.

In such an approach, each pixel can record the time when a preset intensity threshold is exceeded, with nanosecond time resolution, so the timing accuracy of these devices can be much better than the camera frame rate.[20,22] Additionally, only the pixels where the threshold has been exceeded can be read out, either on the ASIC or readout electronics level, reducing the amount of data compared to conventional cameras. Similar to the conventional cameras, which integrate and record the incoming flux, the photon counting cameras determine positions of the events from the hit pixel coordinates.[17]

The Timepix ASIC[23] is a CMOS pixel read-out chip, originally developed at CERN for particle physics applications. It has 256 × 256 pixels and 55 × 55 μm^2 pixel size, and

[a)]Current address: Randall Division of Cell and Molecular Biophysics, King's College London, New Hunt's House, Guy's Campus, London SE1 1UL, United Kingdom.

10 ns timing resolution. This readout chip, after the addition of a bump-bonded silicon sensor, has been previously used for the detection of charged particles and X-rays and applied, for example, to ion imaging.[24,25] For the ion detection, some measurements used bare Timepix chips placed in the vacuum behind an MCP to collect the electrons from the MCP directly. The chip was also used for the detection of visible light in a specialized vacuum device with a photocathode and MCP,[26–28] where metal pads of the Timepix pixels were directly sensing electrons after the MCP.

Recently a new camera, TimepixCam, which employs the Timepix chip in combination with a back-side illuminated silicon sensor, was developed for visible light detection.[29] Here, we have used the TimepixCam camera in combination with an off-the-shelf image intensifier for wide-field time-correlated single photon counting (TCSPC) imaging, to map phosphorescence decays of iridium complexes with lifetimes of ~1 μs. The image intensifier is needed since the sensitivity of TimepixCam by itself is not sufficient to register single photons in the visible with an energy of 2-4 eV. To our knowledge, these are the first TCSPC experiments which involve the imaging of single photons with simultaneous time stamping at the pixel level with 10 ns scale time resolution.

II. METHOD

A. TimepixCam

The camera has been described in detail previously.[29] Briefly, TimepixCam is based on the XRI UNO X-ray camera (X-Ray Imatek S.L., Spain) which has a maximum continuous readout rate of ~10 fps, which is limited by the bandwidth of the USB2 readout. The camera is fitted with a Timepix readout chip, with a specialized silicon sensor bump bonded to the chip. In addition to a thin passivation layer on the topside of the sensor, an anti-reflective coating optimized for 400-450 nm photons was deposited across the sensor. This coating increases sensitivity by decreasing the reflection from 35% for uncoated silicon to about 5% and is optimised for the P47 phosphor emission spectrum. The quantum efficiency of the sensor is above 85% in the 400-900 nm range.[30]

The Timepix ASIC operates in the photon counting mode described above when each pixel has a threshold and the time is measured only when the threshold is crossed. The chip has 256 × 256 pixels, where processing electronics in each pixel amplify and shape the input charge, recording the time of arrival of events which cross a preset threshold. The pixel noise is about 100 electrons and minimal threshold is about 650 electrons.[23] Therefore, for a Timepix pixel connected to the light sensitive sensor described above it would require at minimum a flash of about 700-800 synchronous photons, depending on the wavelength, to cross the threshold and measure time. In practice, the event threshold was set such that almost no pixels were triggered in dark conditions.

The events are stored as a timecode in a 14-bit memory inside each pixel. Only one timecode per pixel can be stored during a single exposure frame. Each pixel also includes a 4-bit digital-to-analog converter, which allows adjustment of the threshold at the individual pixel level using an equalization algorithm. In the experiments described below, the chip was run with a 20 ns clock cycle and a frame exposure time of 200 μs. Timepix does not have data sparsification at the chip level so the whole pixel matrix is read out for each frame. The zero timecodes are then suppressed in the readout electronics before sending the data to the PC.

B. Experimental setup

The experimental setup was built on a Nikon Eclipse TE2000-U inverted microscope, as shown in Fig. 1(a). A Horiba DeltaDiode pulsed diode laser with a 485 nm head (DD-485L, 80 ps pulse width) was used for excitation of the sample. A 475/28 filter was inserted in front of the laser, the beam was expanded and then focused onto the back focal plane of the objective for wide-field illumination. The sample was placed on the microscope sample stage and imaged with a 10× NA0.3 or 4× NA0.13 air objective (Nikon, Japan). The excitation and emission light was separated with a 500 nm dichroic mirror, and a 515 nm long-pass emission

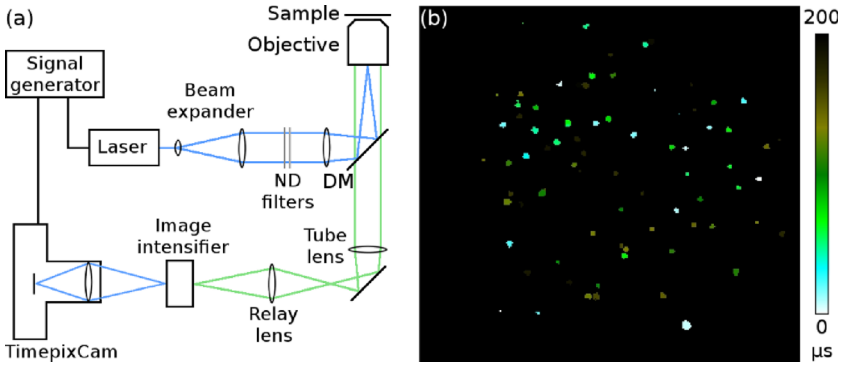

FIG. 1. (a) Schematic diagram of the experimental setup. A 485 nm picosecond pulsed laser was synchronised with the detector using a signal generator. The laser beam was expanded and the intensity was controlled with neutral density (ND) filters. The fluorescence from the sample was collected through a dichroic mirror (DM). The image formed in the microscope image plane was relayed onto the image intensifier, and the intensifier screen was imaged with TimepixCam. (b) Single photon events on the screen of the image intensifier, imaged with TimepixCam during a single frame with an exposure time of 200 μs. The colours indicate the time at which a preset intensity threshold was crossed for each pixel during the frame exposure. Photons were arriving at random times for this measurement.

filter was placed in the emission path. The microscope internal magnification was set to 1.5, and a relay lens (f/1.2, Canon, Japan) was placed between the microscope image port and the detector provided an additional magnification of 3.7.

A 40 mm diameter dual proximity-focused, two-microchannel plate image intensifier with a P47 phosphor screen and S20 photocathode with a peak quantum efficiency of 15% at 510 nm (Photek, U.K.) which operated with saturated gain was placed in the intermediate image plane after the relay lens. The illumination intensity was reduced using neutral density filters such that single photons could be observed on the screen of the image intensifier, see Fig. 1(b). TimepixCam has a 50 mm f/1.2 lens (Canon, Japan) to project the 40 mm diameter phosphor screen onto the 14×14 mm^2 sensitive area of the sensor, providing an image demagnification of 2.9. This optical configuration maps 0.16×0.16 mm^2 of phosphor screen area onto each 55×55 μm^2 TimepixCam pixel.

The TimepixCam and the laser were synchronised using a signal generator (TG105, Thandar, U.K.). The signal repetition rate was set to 300 Hz, and the Timepix electronic shutter was opened with a transistor-transistor logic (TTL) high signal with a 200 μs gate width. The laser was triggered from the "trigger out" connection of the signal generator, such that the laser pulse reached the camera shortly after the shutter was opened. The images were read out from the camera to the PC with ~10 Hz frame rate. The data from the camera were saved into text files, one file per frame, which recorded the x and y positions and the timecode for each pixel that was hit during that frame.

C. Data processing

The lifetime images were created by placing all photons into an xyt data cube and writing the data into an .ics image file with a program written in C (programming language). A monoexponential function $I(t) = I_0 e^{-\frac{t}{\tau}}$, where I_0 is the initial fluorescence intensity and τ is the lifetime, was fitted to the phosphorescence intensity decay in each pixel of the image with Tri2 software.[31] The lifetime obtained from this fit was encoded in a pseudocolor scale (blue for short lifetimes and red for long lifetimes), yielding a fluorescence lifetime image. A fluorescence intensity image was obtained by summing all the raw frames together. For the final result image, each pixel in lifetime image was weighted by the grey value of that pixel in the fluorescence intensity image, yielding an image that shows both the fluorescence lifetime (colour) and the fluorescence intensity (brightness).

D. Sample preparation

For the measurement of the instrument response, a 1951 USAF resolution test chart was overlaid with a fluorescent plastic slide (92001, Chroma, VT) with a fluorescence lifetime of a few nanoseconds.

For phosphorescence lifetime imaging, polystyrene beads (200 μm diameter) were swollen by suspension in dichloromethane and then soaked in dichloromethane solutions of iridium complexes Ir(BMes$_2$)$_2$acac,[32] Ir(ppy)$_3$, and Ir(fppy)$_3$.[33] After soaking for 30 min the leftover liquid was poured away and the beads were washed with ethanol, which caused them to shrink and trap the dyes inside the particles. After thorough washing with ethanol the beads were air dried. The beads were mixed with small pieces of fluorescent plastic and placed on a multiwell plate with #1.5 coverslip bottom for imaging.

III. RESULTS

A. Single photon events and centroiding

To evaluate the detection system response prior to imaging the test pattern and bead samples, the TimepixCam in combination with the image intensifier was illuminated by a faint light source and the resulting performance parameters were evaluated. The images of single photon events on the screen of the image intensifier acquired with TimepixCam are approximately round, with a diameter of a few pixels. Fig. 1(b) shows single photon events acquired with continuous illumination, where the photons arrive at random times and are thus evenly distributed in time. Fig. 2 shows single photon events from a fluorescent sample with a nanosecond decay time, where the excitation laser pulse was synchronised with the camera, and the arrival time distribution of the photons is narrow.

However, the pixels associated with a single photon event have a time distribution themselves, where the centre of the photon event with the highest flux has the earliest time stamp, and the surrounding pixels may have their time stamps delayed by a few timecodes. This effect is due to the lower flux in these pixels as the corresponding pulses have a slower rise time and therefore cross the threshold slightly later, an effect known as "timewalk." As each cluster represents a single photon which has only one arrival time, we have employed a centroiding algorithm, which finds contiguous clusters of four or more triggered pixels, selects the earliest timecode from the cluster,

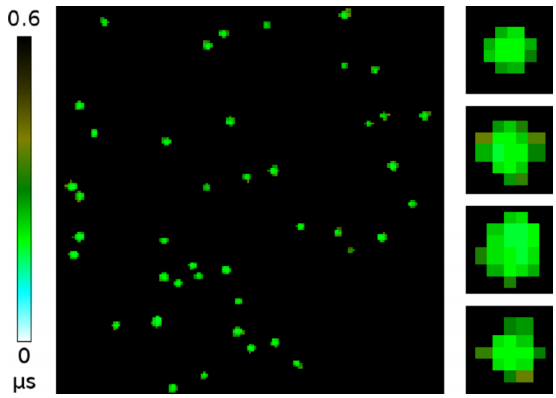

FIG. 2. Single photon events from a fluorescent sample with a fluorescence decay time of a few nanoseconds, where the excitation laser pulse was synchronised with the camera. Image size 180×180 pixels, and enlarged areas of 7×7 pixels for four photon events. The colours indicate the time when a preset intensity threshold was crossed for each pixel during the frame. The average arrival time distribution of the photons is narrow, but the individual photon events themselves have an arrival time distribution.

and assigns this time code to the centre of mass of the event. Previous studies indicate that a significant improvement in both time and spatial resolution is obtained when applying a four pixel threshold to the photon events.[29]

B. Instrument response

To measure the detector time response and spatial resolution, a 1951 USAF resolution test chart was overlaid with a fluorescent plastic slide whose decay time, on the order of a few nanoseconds, is short enough for the measurement of the instrument response. The sample was mounted on the microscope stage and imaged with a 10× objective. A total of 200 000 frames were acquired. Fig. 3 shows the images obtained by summing all frames and the distribution of time codes in two cases: for all time codes (Figs. 3(a) and 3(c)) and for the time codes corresponding to the cluster centroids (Figs. 3(b) and 3(d)). Centroiding, as described above, removes background noise from the image and improves sharpness (Fig. 3(b)) compared to the sum of all time codes (Fig. 3(a)). Centroiding also significantly shortens the time code distribution, as shown in Figs. 3(c) and 3(d). The time resolution was estimated to be 15.5 ns by fitting a Gaussian function to the measured time code distribution in Fig. 3(d).

C. Phosphorescence lifetime imaging

For phosphorescence lifetime imaging, the iridium bead sample was placed on the microscope sample holder and imaged with a 4× objective. Wide-field TCSPC lifetime images of this sample are shown in Fig. 4(a). The dataset consists of 100 000 frames. The different iridium compounds and the fluorescent plastic are distinguishable by lifetime. Phosphorescence decays of small areas indicated in Fig. 4(a) are shown in Fig. 4(b). Monoexponential fits to these datasets yield lifetimes of 427 ± 1 ns for A2, 448 ± 1 ns for A3, and 875 ± 6 ns for A4. The lifetime of the fluorescent plastic in A1, on the order of a few nanoseconds, is too short to be measured with this detector.

IV. DISCUSSION

In this work we have combined the TimepixCam camera with a photon counting image intensifier to perform time-correlated single photon counting imaging. The TimepixCam sensor is sufficiently sensitive to detect the single photon events on the phosphor screen of the image intensifier, which are approximately round clusters with a diameter of a few pixels. Each cluster has a distribution of time codes caused by a fast rise of the intensity in the middle of the cluster and slow rise in the surrounding pixels. As each event corresponds to one photon which only has one arrival time, a centroiding algorithm was employed which assigns the earliest arrival time in the cluster to the centre of mass of the event. This improves the timing resolution of the detector significantly and also improves the image quality by removing some of the blurring caused by the electron amplification process in

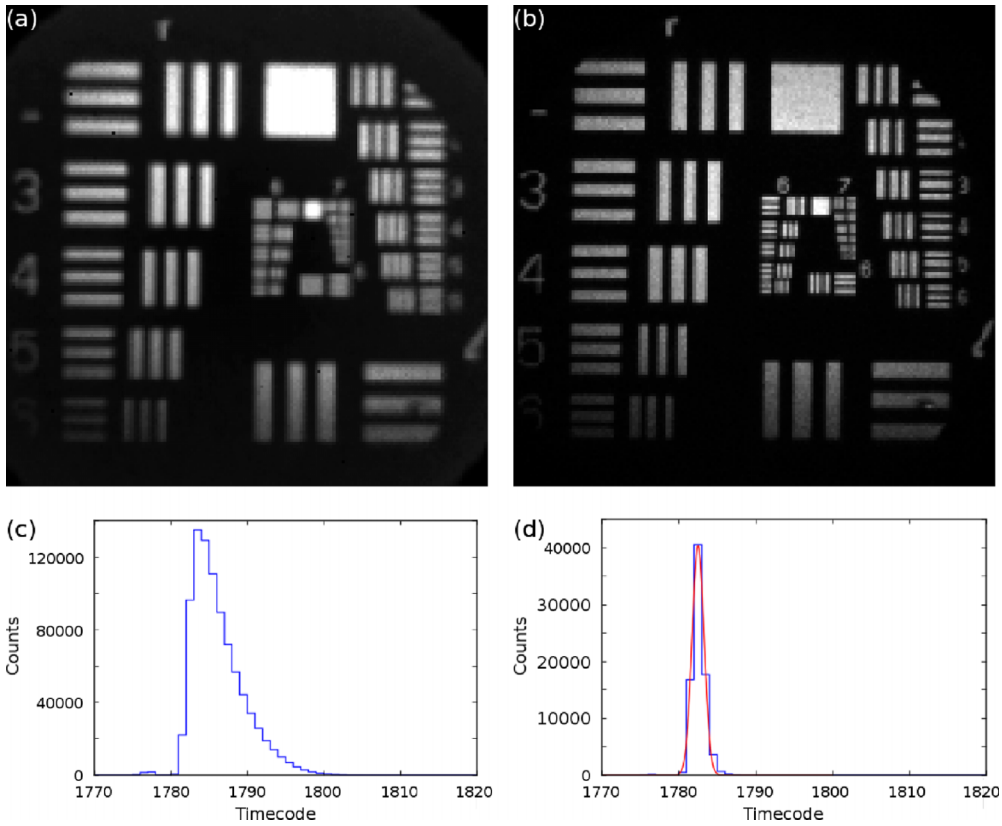

FIG. 3. (a) and (b) Images of USAF test pattern overlaid with fluorescent plastic. (a) The sum of raw frames, (b) centroided image. (c) and (d) Instrument response with (c) all time codes and (d) centroiding. A Gaussian fit (red line) to the measured data in (d) yields a time resolution of 15.5 ns.

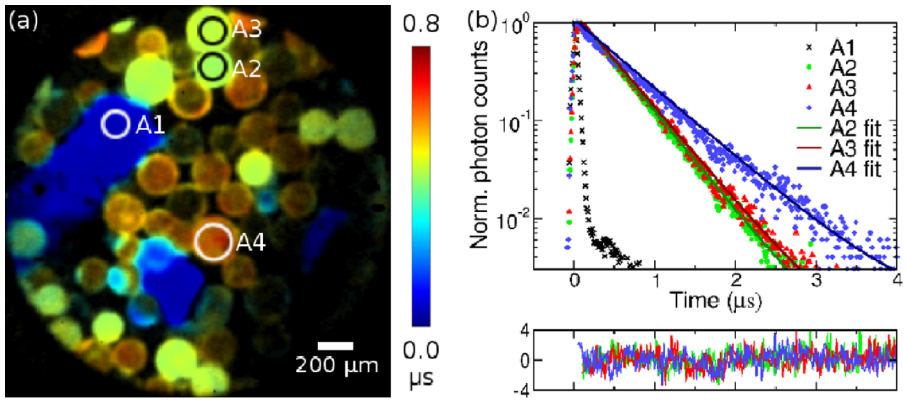

FIG. 4. (a) Lifetime images of beads infused with different Ir-compounds and fluorescent plastic acquired with TimepixCam. (b) Intensity as a function of time (phosphorescence decays) for areas A1-A4 indicated in (a), with monoexponential fits to the phosphorescence decays and residuals of the fits.

the image intensifier. The sensor was used with a 20 ns clock cycle, resulting in an estimated time resolution of 15.5 ns.

Applying this detector system to phosphorescence lifetime imaging (PLIM) microscopy of 200 μm polystyrene beads with iridium complexes shows clear decays over two orders of magnitude of intensity. Fitting these decays with a monoexponential decay function yields a good fit as indicated by the flat residuals in Fig. 4(b), with lifetimes of 427 ± 1 ns, 448 ± 1 ns, and 875 ± 6 ns. The precision, accuracy, and high signal-to-noise ratio of TCSPC allow the close lifetimes 427 ns and 448 ns to be distinguished.

The wide-field TCSPC imaging approach described here is especially well suited for time-resolved imaging in the microsecond regime. It combines exceptionally low excitation powers of fractions of a microwatt with the collection of up to hundreds of photons per excitation cycle. In contrast to sequential gating techniques, no photons are lost. Due to the digital nature of photon counting and its associated advantages—e.g., Poisson statistics, a large dynamic range, a high time resolution, and easy visualization of decays—it also has a better signal-to-noise ratio than frequency modulation techniques at low signal levels. This is an important consideration, first in view of the limited photon budget available from fluorophores before they are irreversibly bleached,[34] and second, to lower the risk photodamage when a living specimen is imaged. Given sufficient photon counts, meaningful double-exponential decay analysis is also feasible.

Alternative wide-field TCSPC techniques include position-sensitive read-out anodes which can reach picosecond time resolution[17,35–37] and are best suited for imaging nanosecond fluorescence decays.[38,39] SPAD arrays can detect individual photons in each pixel with picosecond resolution without an image intensifier, and 256 × 256 pixel devices have been developed.[40] They allow enormous count rates[41] and are very promising single photon sensitive cameras for the future.[42] However, currently their use as cameras is limited by a low fill factor, high noise levels, and nonuniformity across the array.

The main drawback of the current camera at this stage is the 10 fps maximum continuous readout rate, which is limited by the USB bandwidth of the XRI UNO camera. The Timepix chip can be read out at much faster frame rates, up to a few kHz, and work is in progress to implement a new version of TimepixCam with a faster readout. The silicon sensor described above is fully compatible with the new generation of Timepix3 chips,[43] which improve the timing resolution by an order of magnitude to 1.5 ns. Another attractive feature of Timepix3 is the asynchronous readout of the hit pixels with only 0.5 μs dead time, which will effectively allow multi-hit functionality at the pixel level, similar to the Pixel Imaging Mass Spectrometry (PImMS) sensor.[44]

V. CONCLUSION

We have demonstrated phosphorescence lifetime imaging microscopy with a novel optical imager, TimepixCam, that is optimised for visible light detection and read out by a Timepix ASIC, and applied this detector system to lifetime imaging of iridium compounds with lifetimes on the order of a few hundred nanoseconds. The current system is optimal for time-resolved imaging in the hundreds of nanoseconds to microsecond region, with a time bin width of 20 ns that can be improved to 1.5 ns with the Timepix3 ASIC. Imaging of microsecond lifetimes in PLIM is of great interest in the life sciences, for example, for oxygen sensing,[5,6] or imaging viscosity or measuring hydrodynamic radii,[7,8] and has been used in other fields to study air-flow and pressure in aerodynamic studies,[9] and for temperature measurements in industrial thermometry applications.[10] The TimepixCam allows imaging for these applications at the photon counting level with nanosecond time resolution.

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